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Award Number: DAMD17-01-1-0570

TITLE: Control of Metastasis-Associated Gene Expression by Cell-surface Beta-1,6 Branched Oligosaccharide Levels

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REPORT DATE: June 2003

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Final Addendum (1 Jun 2002 - 31 May 2003)

4. TITLE AND SUBTITLE
Control of Metastasis-Associated Gene Expression by Cell-surface Beta-1,6 Branched Oligosaccharide Levels

5. FUNDING NUMBERS
DAMD17-01-1-0570

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Metastatic breast cancer cells expresss cell-surface Beta-1,6 branched oligosaccharide structures, which are reduced/absent in normal breast tissue. The biosynthesis of these structures ininitiated by the Golgi-localized glycosyltransferase N-Acetylglucosmaminyl-transferase V (GlcNAc-T V). The altered expression of GlcNAc-T V in animal mammary cancer models also influences metastasis. Furthermore, altered transcription of metastasis-associated genes has also been observed incells with a reduced expression of beta-1,6 branched oligosaccharide expression.

The aim of this project is to characterize the altered expression of mRNA in mouse and human mammary cancer-derived cell lines as a function of altered Beta-1,6 branched oligosaccharide expression. This will be accomplished by using DNA Microarray technology to assess the mRNA levels in cell lines transfected with GlcNAc-T V expression vectors. After identification of mRNA molecules that are altered by changes (elevations or reduction) in cell-surface Beta-1,6 branched oligosaccharide expression, the kinetics of induction will be characterized using cell lines with GlcNAc-T V under the control of an inducible promoter.

14. SUBJECT TERMS
Oligosaccharides, Glcycosyltranferases, DNA Microarrays, Gene Expression, Metastasis

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Addendum to final report

Introduction

This addendum to the final report of the above referenced grant describes continued experiments on the project. Further studies on the effects on mRNA expression of inducing the expression of the glycosyltransferase N-Acetylglucosaminyltransferase V (GlcNAc-T V) in murine mammary cancer cells were performed. The University of Nebraska Medical Center Microarray Core Facility has prepared a new, expanded set of microarray chips, containing 15,000 murine cDNA's. These chips were probed for differences in expression between the control transfected cel line 410.4-C6 and the 410.4-TV.84 cell line, which had been stably transfected with a GlcNAc-T V expression vector and which was expressing elevated levels of GlcNAc-T V. To examine the validity of the microarray data, PCR primers to selected mRNA's were designed in the laboratory and used to determine the level of mRNA present in the RNA preparations. By comparison to internal controls, the relative levels of mRNA between the 410.4-TV.84 and the 410.4C6 cell lines could be compared.

Results

Total RNA was purified from the 410.4-C6 and the 410.4-TV.84 cell lines as described previously, labeled with either Cy3 or Cy5 dyes, combined, and hybridized to the Microarray slides. Technical replicates of paired RNA preparations isolated from both cell lines on the same day and also on independently isolated samples prepared on a different day from the same cell lines were performed. Both elevated and decreased gene expression was observed in the transfected cell line. The cut-off for inclusion in this table was a two-fold difference for both elevated and reduced levels of gene expression. Table 1 shows the genes that are elevated in the 410.4-TV.84 cell line RNA preparations. Table 2 shows those genes that are decreased in the 410.4-TV.84 cell line. Note that a 0.50 value in Table 2 corresponds to a two-fold decrease in RNA expression.

Table 1. Genes with Elevated Expression in 410.4-TV.84 cells

Gene Name	I.D. No.	Microarray Ratio
Placental and Embryonic Early Expression Gene (PEM)	BG078428	36.7
Thymic Shared Antigen (TSA-1)	BG078395	12.4
Secreted Acidic Cysteine-rich Glycoprotein (Sparc)	BG064802	7.5
Heat Shock Protein 75	BG078439	2.3
Unknown	BG082965	12.6
FK506 Binding Protein 4	BG069755	2.2
Cyclin D1	BG083088	3.3
MilP (mitochondrial)	BG075262	3.2
High Mobility Group Protein 2	BG085427	3.0
47 kDa Heat Shock Protein (HSP47)	BG086364	4.1
Myocardium Phospholipid Hydroperoxide Glutathione	AW546565	2.0

Table 2. Genes with Decreased Expression in 410.4-TV.84 cells

Gene Name	I.D. No.	Microarray Ratio
U8	BG063815	0.45
Unknown	BG065289	0.50
Phosphoribosyl Pyrophosphate Synthetase I	BG064866	0.50
Phosphoglycerate kinase processed pseudogene	BG064745	0.45
MORF-related gene X	BG063728	0.47
Unknown	BG065373	0.47
N-myc downstream Regulated (Ndr1)	BG078732	0.43
Keratin 19	BG064706	0.36
Unknown	C77369	
DKFZP564B167 protein	BG082594	0.50
Nef-associated factor 1	BG069325	0.45
Nras	BG069766	0.50
Caveolin-1, beta isoform	BG083456	0.50
eIF-1A	BG087376	0.49
63 kDa endoplasmic reciculum transmembrane protein	BG086493	0.45
Osteoblast protein (GS3786)	BG086882	0.43
Glucocorticoid-dependent protein kinase	BG072439	0.49
Peroxiredoxin 4 (Prdx4)	BG075324	0.48
Deleted in polyposis 1 (Dp1)	BG072251	0.50
Unknown	BG075693	0.40
Kcnq-1 protein	BG075757	0.21

In order to evaluate the validity of the changes in mRNA expression obtained from the microarray experiments reported in Tables 1 and 2, PCR primer pairs were designed in the lab for four selected mRNA species and used to determine the level of expression by this independent method of measuring the relative levels of mRNA present. Both gene products showing elevated and decreased expression in the Glc-NAc-T V-transfected cells (410.4-TV.84) vs the control (410.4-C6) transfected cells were analyzed. As an internal control to enable comparison between the RNA preparations isolated from the two different cell lines, PCR primers for GAPDH were also used with RNA from both the control-transfected and the GlcNAc-T V-transfected cell lines. Relative ratio's between the experimental and the control mRNA were calculated and use for comparison. The same RNA preparations used in the microarray experiments were used in these analyses.

First a cDNA product was prepared from the RNA preparations using oligo-d(T) as a primer. Following that, twenty rounds of PCR were performed to enable the synthesis of the PCR products. After separation on a 1% agarose gel, the PCR products were visualized with the DNA-intercalating dye, ethidium bromide. Relative amounts of PCR product were quantitated by densitometry and the ratio of experimental to control (GAPDH) calculated for both RNA preparations. The ratios were then compared to the microarray results found in Tables 1 and 2. In all cases the PCR-determined relative expression of genes corresponded well with that observed by microarray analysis, although the absolute measurements were

different. This thus validates the microarray studies as a means of measuring changes in gene expression in these experiments.

Table 3. Comparison of Relative PCR and Microarray Gene Expression Measurements

Gene Name	I.D. No.	Microarray Ratio	PCR Ratio
Placental and Embryonic Early Expression Gene (PEM)	BG078428	36.7	9.5
Thymic Shared Antigen (TSA-1)	BG078395	12.4	4.4
Secreted Acidic Cysteine-rich Glycoprotein (Sparc)	BG064802	7.5	5.1
Keratin 19	BG064706	0.36	0.41
N-myc downstream Regulated (Ndr1)	BG078732	0.43	0.31

Considerable attempts to transfect the GlcNAc-T V expression vector into MCF-7 cell lines met with failure. Although transfection was possible, as measured by the ability to select and clone G-418-resistant cell lines, the levels of Beta-1,6 branched oligosaccharide levels was unchanged when compared to parental or control-tranfected (vector only) cell lines. This lack of change in oligosaccharide expression was determined by lectin-blot analysis of SDS-PAGE separated membrane glycoproteins using L-PHA, the plant lectin reactive with Beta-1,6 branched oligosaccharides.

Discussion

The results of these studies have demonstrated that the increase of the expression of cell-surface Beta-1,6 branched oligosaccharides as a result of the transfection of GlcNAc-T V, the glycosyltransferase that initiates this branch, results in an alteration in the expression of different genes, both with elevations and reductions in gene expression. Some of the genes found to be elevated in this study in the transfected cells, which have already been shown to have increased metastatic properties, are associated with increased tumorigenic/metastatic properties. Elevated expressions of the PEM, TSA-1, Sparc, and cyclin D1 genes are all found in tumors. Likewise, a reduction in cytokeratin 19 gene expression is found in tumors.

Of concern is that when compared to the first experiments performed with a different microarray slide set, only cyclin D1 was consistently identified as being changed (elevated) in both sets. This raises a concern as to the reliability of the previous data set, particularly since the changes in the gene expression reported above in Tables 1 and 2 have confirmed by PCR analysis for the five genes reported in Table 3. PCR analysis of cyclin D1 is planned, as are analysis of selected genes that were reported to have only 2-3-fold elevation in Table 1.

Our hypothesis that alteration of cell-surface Beta-1,6 branched oligosaccharide expression leads to altered gene expression has been proved by complementary studies by microarray and PCR analytical studies. Future studies will concentrate on possible common regulatory motifs in the DNA upstream from the transcriptional start sites of these genes. These will be initially performed by comparing the sequence of these regions of the DNA. If common transcriptional regulatory protein binding sites are observed, the presence of

levels of the transcription proteins will be determined in nuclear extracts of cells.